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Award Number: DAMD17-03-1-0322

TITLE: Investigating the Role of Celecoxib as a
Chemopreventive and Chemotherapeutic Agent for
Breast Cancer

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REPORT DATE: May 2004

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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20040830 091

REPORT DOCUMENTATION PAGEForm Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE May 2004	3. REPORT TYPE AND DATES COVERED Annual Summary (1 May 2003 - 30 Apr 2004)	
4. TITLE AND SUBTITLE Investigating the Role of Celecoxib as a Chemopreventive and Chemotherapeutic Agent for Breast Cancer			5. FUNDING NUMBERS DAMD17-03-1-0322	
6. AUTHOR(S) Randy Levitt			8. PERFORMING ORGANIZATION REPORT NUMBER	
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9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited				12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) Experimental and epidemiological studies have suggested a role for the use of cyclooxygenase(COX)-2 inhibitors in the prevention of breast cancer. The relative lack of toxicity associated with these compounds favors their use as chemopreventive agents, but the underlying mechanism of their chemopreventive effect remains unclear. We have observed that the COX-2 inhibitor celecoxib inhibits growth and induces apoptosis in the immortalized breast epithelial cell line 184htert. Microarray gene expression analysis of 184htert cells treated with 50 μ M celecoxib revealed the modulation of several genes of interest, including a significant induction of expression of the mRNA encoding insulin-like growth factor binding protein-3 (IGFBP-3). IGFBP-3 is a potent pro-apoptotic protein and growth inhibitor of breast cancer cells, which acts mainly by inhibiting the access of the mitogens IGF-I and IGF-II to their cell surface receptor, but also via IGF-independent effects. Quantitative real-time RT PCR demonstrated that 50 μ M celecoxib induced a ~3 fold increase in expression of IGFBP-3 mRNA. Furthermore, ligand blot analysis revealed that celecoxib treatment was associated with the upregulation of IGFBP-3 at the protein level. IGFBP-3 treatment of 184htert cells inhibited IGF-I and serum-induced proliferation, but had no effect on cell growth under serum-free conditions, indicating that IGF-independent effects of IGFBP-3 are not observed in this system. Our results suggest that celecoxib may decrease IGF-I associated breast cancer risk by a mechanism involving induction of expression of IGFBP-3 and subsequent reduced proliferation of at-risk breast epithelial cells.				
14. SUBJECT TERMS COX-2, IGF-I, IGFBP-3, apoptosis, celecoxib				15. NUMBER OF PAGES 24
				16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

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Introduction

A large body of data has suggested a role for non-steroidal anti-inflammatory drugs (NSAIDs) in the treatment and/or prevention of various cancers. The classic actions of NSAIDs involve inhibition of the rate-limiting enzyme in the prostaglandin synthesis cascade, cyclooxygenase (COX). This enzyme exists in two isoforms: COX-1 and COX-2. The expression of COX-1 is constitutive in most tissues whereas the expression of COX-2 is inducible by cytokines, growth factors and tumor promoters (1). NSAIDs have also been shown to act on targets other than the COX enzyme, such as PPAR γ (2) and 3'-phosphoinositide-dependent protein kinase-1 (3).

Several epidemiological studies have demonstrated an inverse relationship between the relative risk of breast cancer and the regular intake of NSAIDs (4,5). However, chronic use of NSAIDs results in gastrointestinal tract toxicity. It is believed that this toxicity is a result of inhibition of the COX-1 isoform (6), and due to their relative lack of toxicity, COX-2 selective NSAIDs (COX-2 inhibitors) are widely considered as potential chemopreventive agents.

There are several reports which associate COX-2 inhibition with breast cancer prevention. Harris et al. have shown that administration of the COX-2 inhibitor celecoxib to DMBA-treated rats produced significant reductions in the incidence, multiplicity, and volume of breast tumors relative to the control group (7). Furthermore, Liu et al. have shown that overexpression of COX-2 is sufficient to induce mammary tumorigenesis in transgenic mice (8).

Despite the epidemiological and experimental evidence indicating that COX-2 inhibitors can act as chemopreventive agents for breast cancer, the exact mechanism remains unclear. The objective of this study was to investigate the effects of the COX-2 inhibitor celecoxib on pre-malignant breast epithelial cells. For these experiments, we used an immortal breast epithelial cell line called 184htert (gift from Dr. Sandra Dunn, University of British Columbia). We show that celecoxib induces growth inhibition and apoptosis in 184htert cells and characterize associated changes in gene expression using microarray analysis. Our array data revealed the modulation of several genes of interest, including a significant induction for the mRNA encoding insulin-like growth factor binding protein-3 (IGFBP-3).

The IGFBPs are a family of 7 proteins (IGFBP-1 to 7) that, depending on the tissue type, are known to either enhance or inhibit IGF action (reviewed in Ref. 9). IGFBP-3 is the major circulating IGFBP, produced mainly by the liver but also a number of tissues produce it locally. IGFBP-3 sequesters the mitogens IGF-I and IGF-II, preventing their binding to the IGF-I receptor, and therefore inhibiting IGF-induced growth (9). As well, IGFBP-3 has been reported to have IGF-independent growth inhibitory effects (10). We demonstrate the induction of IGFBP-3 by celecoxib at the mRNA and protein level, and go on to show that IGFBP-3 antagonizes the growth of 184htert cells in an IGF-dependent manner.

Body

The 184htert cell line is an immortal breast epithelial cell line that was created by transfecting a retrovirus expressing the human telomerase reverse transcriptase gene into normal breast epithelial cells (11). In order to confirm that these cells were immortal and not transformed, we grew them in soft agar for over 3 weeks and they did not form colonies, whereas the MCF-7 breast cancer cell line grown in parallel did form colonies (data not shown). This result is consistent with those of Toouli et al. who observed that htert-immortalized breast epithelial cells did not form tumors in nude mice (12). Therefore, the 184htert cell line is a reasonable system to study the early steps in mammary carcinogenesis. Furthermore, since we detected COX-2 protein expression in these cells (data not shown), 184htert was a suitable cell line to study the chemopreventive effects of the COX-2 inhibitor celecoxib.

To determine if celecoxib could inhibit the growth of these cells, they were treated with either 25 or 50 μ M celecoxib for up to 48 hours. As seen in **Fig. 1a** (Appendix I: Levitt, R.J. et al., 2004), celecoxib inhibited the growth of 184htert cells, with the 50 μ M dosage being significantly more potent than the 25 μ M dosage (~30% growth inhibition by 25 μ M dose after 48 hours, whereas virtually all cells were non-viable after 24 hours with the 50 μ M dose). The observed difference in the potency between 25 and 50 μ M celecoxib was similar to data we had previously obtained with celecoxib treatment of BxPC-3 pancreatic cancer cells (13). In that system, the difference in potency between the two doses of celecoxib could be attributed to the fact that 50 μ M dose induced apoptosis whereas the 25 μ M dose induced G1 arrest. In order to see if this situation was paralleled in 184htert cells, cell cycle analysis was performed on celecoxib treated cells. After 6 hours treatment, 50 μ M celecoxib induced apoptosis in 184htert cells (~15% in sub-G1 population after 6 hours treatment, **Fig.1b**, panel 3), whereas no apoptosis was observed for cells treated with 25 μ M celecoxib (**Fig.1b**, panel 2).

Having established that 50 μ M celecoxib could inhibit growth and induce apoptosis in 184htert cells, we wished to clarify the mechanism underlying this chemopreventive effect. To do so, differential gene expression was evaluated by comparing 184htert cells treated with or without 50 μ M celecoxib for 6 hours using the Affymetrix U95 cDNA microarray. Results from the microarray analysis of celecoxib-induced changes in gene expression are listed in **Tables 1, 2, 3** (Appendix I: Levitt, R.J. et al., 2004). Evidence for the validity of the gene expression data includes the strong upregulation of COX-2 expression by celecoxib treatment (~30-fold induction, **Table 1**), an observation that we confirmed at the protein level (data not shown) and that has been reported in other systems using various NSAIDs (14,15).

Our lab is particularly interested in the association between IGF physiology and breast cancer as IGF-I is known to be a potent mitogen for breast epithelial cells (16), and serum IGF-I levels have been positively correlated with premenopausal breast cancer risk (17). **Fig.2** (Appendix I: Levitt, R.J., 2004) is a scatterplot of the microarray data where each dot represents a probe that detected a statistically significant change in gene

expression induced by celecoxib treatment ($P < 0.05$). Probes that correspond to genes whose products are involved in IGF physiology are labeled. A treatment/control signal ratio (fold change) of ≥ 2 or ≤ 0.5 was considered as a significant induction or repression, consistent with previous reports of microarray gene expression analysis (18,19). Our array data shows that celecoxib did not significantly alter ($0.5 > \text{fold change} > 2.0$) the expression of the IGF-I receptor, IGF-II receptor, nor IGFBPs-2 and 4 (Fig. 2). Furthermore, celecoxib-induced changes in the expression of the ligands IGF-I and IGF-II, and IGFBPs-1,5,6,7 were not statistically significant ($P < 0.05$, data not shown). However, as seen in Fig. 2, the expression of IGFBP-3 was significantly upregulated by celecoxib treatment (~ 20 -fold induction by hybridization to the 37319_at probe, and ~ 4 -fold induction by hybridization to the 1586_at probe). IGFBP-3 is the major circulating IGFBP and it has been shown to inhibit IGF-induced growth and also to have IGF-independent growth inhibitory effects (9,10). Therefore, we hypothesized that IGFBP-3 may be an important modulator of celecoxib's chemopreventive effects. Consistent with this hypothesis, two different probes detected a ~ 0.3 -fold decrease in the expression of protease, serine, 11(IGF binding) (Fig. 2). This protein, which is also known as L56 and HtrA1, has been suggested to be a protease which cleaves and inactivates IGFBPs (20). Thus, celecoxib appears to not only upregulate IGFBP-3 mRNA abundance, but it also decreases the expression of an IGFBP protease.

In order to confirm the induction of IGFBP-3 mRNA by celecoxib, quantitative real-time RT PCR was performed on untreated and celecoxib-treated cells. As seen in Fig. 3a (Appendix I: Levitt, R.J. et al., 2004), an ~ 3 -fold induction in IGFBP-3 mRNA was detected after 6 hours treatment with 50 μM celecoxib. This experiment was performed in serum-free, phenol-red free (SPF) media in order to prevent other factors (estrogen, for example) from interfering with IGFBP-3 promoter activity. To determine if the celecoxib-induced increase in IGFBP-3 mRNA correlated to an increase in IGFBP-3 protein, conditioned media from 184htert cells treated or untreated with celecoxib were assayed by ^{125}I -IGF-I Western ligand blotting. As seen in Fig. 3b, we detected a weak doublet band at ~ 40 -45 kDa in SPF conditioned media under control conditions for 24 hours, and treatment with 50 μM celecoxib resulted in a significant increase in the intensity of this doublet. Therefore, celecoxib treatment of 184htert cells is associated with up-regulation of both IGFBP-3 mRNA and protein. The induction of IGFBP-3 by celecoxib is consistent with the results of Pold et al., who have shown that IGFBP-3 mRNA and protein levels were significantly decreased in A549 human lung cancer cells transduced with COX-2 when compared to the wild-type cell line (21). Taken together, these data lead to the working hypothesis that IGFBP-3 expression is inversely correlated with COX-2 activity.

We wished to determine if the induction of IGFBP-3 by celecoxib in 184htert cells is COX-2 dependent, because although the classic action of celecoxib is to inhibit COX-2 activity, several non-COX targets have been reported (2, 3). Prostaglandin E2 (PGE2) is the major product of the COX-2 pathway, so we investigated whether PGE2 modulates IGFBP-3 expression in 184htert cells. As seen in fig. 5 (Appendix II), treatment of 184htert cells with various concentrations of PGE2 for up to 72 hours had no

effect on IGFBP-3 expression. This data suggests that the induction of IGFBP-3 by celecoxib is mediated by a non-COX target.

To confirm our findings, we treated the COX-2 null breast cancer cell line MCF-7 with 50 μ M celecoxib and assayed for changes in IGFBP-3 mRNA expression after 6 hours treatment. Although treatment with 50 μ M celecoxib resulted in complete cell death after 24 hours, the expression of IGFBP-3 mRNA after 6 hours treatment unexpectedly decreased compared to the control condition, as assayed by quantitative real-time RT-PCR (data not shown). IGFBP-3 is a known inhibitor of MCF-7 cell growth (9), so the fact that its expression is decreased by celecoxib is surprising considering that celecoxib is a potent growth inhibitor of this cell line. This result leads us to believe that IGFBP-3 is not involved in the mechanism of celecoxib-induced growth inhibition of MCF-7 cells, however we wished to further define the role of IGFBP-3 induction in celecoxib-induced growth inhibition of 184 htert cells.

We first looked for a direct growth inhibitory effect of IGFBP-3 on 184 htert cells, as published results on the effect of IGFBP-3 on non-malignant breast epithelial cell growth have been inconsistent. Martin et al. have shown that doses ranging from 10-100 ng/ml of IGFBP-3 can inhibit the growth of the immortal breast epithelial cell line MCF-10a in an IGF-independent fashion (22), however McCaig et al. have demonstrated a biphasic response of MCF-10a cells to IGFBP-3 under serum-free conditions, with low-doses (~20 ng/ml) acting as growth inhibitors and high doses (100-200 ng/ml) acting as growth promoters (23). Furthermore, Strange et al. have shown that 50-500 ng/ml IGFBP-3 can inhibit both IGF-dependent and independent growth of normal human mammary epithelial cells in a dose-dependent manner (16).

When we treated 184 htert cells with 500 ng/ml IGFBP-3 for 72 hours in SPF media, no significant effect on cell growth was observed (**Fig. 4a**, Appendix I: Levitt, R.J. et al., 2004). Treatment with lower doses of IGFBP-3 (25-100 ng/ml) for up to 72 hours yielded similar results (data not shown), suggesting that 184 htert cells do not display any IGF-independent growth inhibition by IGFBP-3. The fact that we did not observe the IGF-independent effects of IGFBP-3 that were reported in other breast epithelial cell lines might be due to the transfected telomerase in 184 htert cells, as telomerase activity in breast epithelial cells has been associated with resistance to at least two other growth inhibitors, namely topoisomerase inhibitors (24) and transforming growth factor beta (25). It should be noted that our array data detected a celecoxib-induced 0.3-fold decrease in the expression of telomerase reverse transcriptase ($P=0.0003$), potentially antagonizing its protective effect against growth inhibition by celecoxib. This may explain why celecoxib is such a potent growth inhibitor in this system. We plan to assay the modulation of IGFBP-3 expression by celecoxib in a breast epithelial cell line which does not express the telomerase reverse transcriptase, namely MCF-10a.

Since IGFBP-3 did not affect the growth of 184 htert cells under serum-free conditions, its upregulation is most likely not directly contributing to the celecoxib-induced growth inhibition observed in Fig. 1a. However, upregulation of IGFBP-3 by

celecoxib may contribute to its chemopreventive action by suppressing mitogenic and anti-apoptotic signals induced by IGF-I. To test this hypothesis, 500 ng/ml IGFBP-3 was added to 184hTert cells in the presence of 50 ng/ml IGF-I. As seen in **fig.4a**, 500ng/ml IGFBP-3 was capable of significantly attenuating an IGF-I-induced increase in cell growth (~70% decrease, IGFBP-3 and IGF-I vs. IGF-I alone). Furthermore, when 500 ng/ml IGFBP-3 was added to cells growing in 5% fetal bovine serum (FBS), ~20% growth inhibition was observed (**Fig. 4b**), potentially due to IGFBP-3 blocking the actions of the IGF-I present in FBS. These data indicate that IGFBP-3 can significantly attenuate IGF-induced growth in 184hTert breast epithelial cells, which is consistent with published results using primary breast epithelial cells (16).

In order to further clarify the contribution of IGFBP-3 to the growth inhibitory properties of celecoxib, we are currently in the process of finding a suitable antisense technology to downregulate IGFBP-3 expression. Although we had originally planned to design our own antisense oligonucleotides or short interfering (si)RNA towards IGFBP-3, the company Dharmacon has just recently made available an IGFBP-3 siRNA for commercial purchase. We have already used commercial siRNA from Dharmacon to downregulate the expression of other IGFBPs in other systems by over 70%, so we are confident that their IGFBP-3 siRNA will be a useful tool for our study. Furthermore, we have formed a collaboration with Merck and Co. to confirm our results obtained to date with celecoxib using their COX-2 inhibitor rofecoxib. Due to our observation that the induction of IGFBP-3 by celecoxib is due a non-COX target, it will be of great interest to determine if rofecoxib can also induce IGFBP-3 expression in breast epithelial cells.

Although we do not plan to further investigate the specific non-COX target that mediates the induction of IGFBP-3 by celecoxib in the 184hTert cell line, we speculate that celecoxib upregulates IGFBP-3 by activating PPAR γ . Our rationale is that celecoxib has been shown to activate PPAR γ (2) and recently, a protein called CITED2 has been shown to be a coregulator of PPAR γ (28). Examining the transcriptional activators modified by celecoxib-treatment in our microarray experiment revealed a 3.1-fold increase ($P=0.00003$) in the expression of CITED2. Interactions among CITED2, AP-2, and p300/CBP are necessary for AP-2 mediated transcriptional activation (27), and since both AP-2 and p300 binding sites are located within the IGFBP-3 promoter (26), celecoxib-induced IGFBP-3 expression may be at least partially due to PPAR γ and CITED2 activation.

Key Research Accomplishments

- 1) Determined that the COX-2 inhibitor celecoxib upregulates IGFBP-3 mRNA and protein in the breast epithelial cell line 184htert.
- 2) Determined that this upregulation is not due to the inhibition of PGE2 (the major product of the COX-2 enzyme), and is therefore likely modulated by a non-COX target.
- 3) Determined that celecoxib unexpectedly decreased IGFBP-3 expression in the MCF-7 breast cancer cell line.
- 4) Determined that IGFBP-3 can inhibit IGF-I and serum-induced proliferation of 184htert cells, but had no effect under serum-free conditions, indicating that IGF-independent effects of IGFBP-3 are not observed in this system.

Reportable Outcomes

A) Manuscripts (copy attached as Appendix I)

- 1) **Levitt, R.J.**, Buckley, J., Blouin, M.J., Schaub, B., Triche, T. and Pollak, M. Growth inhibition of breast epithelial cells by celecoxib is associated with upregulation of insulin-like growth factor binding protein expression. *Biochemical and Biophysical Research Communications* 316: 421-428, 2004.

B) Abstracts and poster presentations

- 1) **Levitt, R.J.**, Buckley, J., Blouin, M.J., Schaub, B., Triche, T. and Pollak, M. Celecoxib induces insulin-like growth factor binding protein-3 mRNA in breast epithelial cells – relevance to chemoprevention? BioContact 2003 (Quebec City, Quebec, Canada, October 2003). ***One of 12 national finalists for Next Generation CIHR/BioContact-Québec 2003.**
- 2) **Levitt, R.J.**, Buckley, J., Blouin, M.J., Schaub, B., Triche, T. and Pollak, M. Growth inhibition of breast epithelial cells by celecoxib is associated with upregulation of insulin-like growth factor binding protein expression. Canadian Breast Cancer Research Alliance Reasons for Hope Conference (Ottawa, Ontario, Canada, October 2003).

C) Funding obtained based on work supported by this award

- 1) Merck Medical School Grants Committee: \$43,968 CDN to cover materials and supplies to investigate the role of rofecoxib (VioxxTM) in breast cancer treatment and prevention.

Conclusions

In this report, we show that the COX-2 inhibitor celecoxib can inhibit growth and induce apoptosis in 184htert breast epithelial cells. Gene expression analysis using cDNA microarrays revealed that the effects of celecoxib are associated with expression changes in many genes of interest, consistent with the view that COX-2 inhibitors have multiple mechanisms of action as cancer chemopreventive agents. Our array data demonstrated that celecoxib-induced the expression of IGFBP-3, an observation we confirmed at the mRNA and protein levels. The addition of PGE2 did not effect IGFBP-3 expression, suggesting that celecoxib's regulation of IGFBP-3 is via the modulation of a non-COX target. The fact that IGFBP-3 can attenuate the growth promoting effects of IGF-I in this experimental system raises the possibilities that: 1) celecoxib may decrease breast cancer risk by a mechanism involving IGFBP-3 induction, acting to slow proliferation of at-risk breast epithelial cells, and 2) the risk reduction associated with COX-2 inhibitors may not be homogenous in populations, but rather be particularly useful in individuals with higher serum IGF-I levels.

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Appendices

Appendix I: Levitt et al., 2004

Appendix II : Figure 5



Growth inhibition of breast epithelial cells by celecoxib is associated with upregulation of insulin-like growth factor binding protein-3 expression[☆]

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Received 6 February 2004

Abstract

Several experimental and epidemiological studies have suggested a role for the use of cyclooxygenase (COX)-2 inhibitors in the prevention of breast cancer. The relative lack of toxicity associated with these compounds favors their use as chemopreventive agents, but the underlying mechanism of their chemopreventive effect remains unclear. We have observed that the COX-2 inhibitor celecoxib inhibits growth and induces apoptosis in the immortalized breast epithelial cell line 184htert. Microarray gene expression analysis of 184htert cells treated with 50 μ M celecoxib for 6 h revealed the modulation of several genes of interest, including a significant induction of expression of the mRNA encoding insulin-like growth factor binding protein-3 (IGFBP-3). IGFBP-3 is a potent pro-apoptotic protein and growth inhibitor of breast cancer cells, which acts mainly by inhibiting the access of the mitogens IGF-I and IGF-II to their cell surface receptor, but also via IGF-independent effects. Quantitative real-time RT PCR demonstrated that 50 μ M celecoxib induced a \sim 3-fold increase in expression of IGFBP-3 mRNA after 6 h. Furthermore, ligand blot analysis revealed that celecoxib treatment was associated with the upregulation of IGFBP-3 at the protein level. IGFBP-3 (500 ng/ml) treatment of 184htert cells inhibited IGF-I and serum-induced proliferation, but had no effect on cell growth under serum-free conditions, indicating that IGF-independent effects of IGFBP-3 are not observed in this system. Our results suggest that celecoxib may decrease IGF-I-associated breast cancer risk by a mechanism involving induction of expression of IGFBP-3 and subsequent reduced proliferation of at-risk breast epithelial cells.

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Keywords: IGF-I; IGFBP-3; COX-2; Celecoxib; Chemoprevention; cDNA microarray

A large body of data has suggested a role for non-steroidal anti-inflammatory drugs (NSAIDs) in the treatment and/or prevention of various cancers. The classic actions of NSAIDs involve inhibition of the rate-

limiting enzyme in the prostaglandin synthesis cascade, COX. This enzyme exists in two isoforms: COX-1 and COX-2. The expression of COX-1 is constitutive in most tissues, whereas the expression of COX-2 is inducible by cytokines, growth factors, and tumor promoters [1]. NSAIDs have also been shown to act on targets other than the COX enzyme, such as PPAR γ [2] and 3'-phosphoinositide-dependent protein kinase-1 [3].

Several epidemiological studies have demonstrated an inverse relationship between the relative risk of breast cancer and the regular intake of NSAIDs [4,5]. However, chronic use of NSAIDs results in gastrointestinal

[☆] Supplementary data associated with this article can be found at doi:10.1016/j.bbrc.2004.02.062.

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tract toxicity. It is believed that this toxicity is a result of inhibition of the COX-1 isoform [6], and due to their relative lack of toxicity, COX-2 selective NSAIDs (COX-2 inhibitors) are widely considered as potential chemopreventive agents.

There are several reports which associate COX-2 inhibition with breast cancer prevention. Harris et al. [7] have shown that administration of the COX-2 inhibitor celecoxib to DMBA-treated rats produced significant reductions in the incidence, multiplicity, and volume of breast tumors relative to the control group. Furthermore, Liu et al. [8] have shown that overexpression of COX-2 is sufficient to induce mammary tumorigenesis in transgenic mice.

Despite the epidemiological and experimental evidence indicating that COX-2 inhibitors can act as chemopreventive agents for breast cancer, the exact mechanism remains unclear. The objective of this study was to investigate the effects of the COX-2 inhibitor celecoxib on pre-malignant breast epithelial cells. For these experiments, we used an immortal breast epithelial cell line called 184htert (generous gift from Dr. Sandra Dunn, University of British Columbia). We show that celecoxib induces growth inhibition and apoptosis in 184htert cells and characterize associated changes in gene expression using microarray analysis. Our array data revealed the modulation of several genes of interest, including a significant induction for the mRNA encoding insulin-like growth factor binding protein-3 (IGFBP-3).

The IGFBPs are a family of seven proteins (IGFBP-1 to 7) that, depending on the tissue type, are known to either enhance or inhibit IGF action (reviewed in [9]). IGFBP-3 is the major circulating IGFBP, produced mainly by the liver but also a number of tissues produce it locally. IGFBP-3 sequesters the mitogens IGF-I and IGF-II, preventing their binding to the IGF-I receptor, and therefore inhibiting IGF-induced growth [9]. As well, IGFBP-3 has been reported to have IGF-independent growth inhibitory effects [10]. We demonstrate the induction of IGFBP-3 by celecoxib at the mRNA and protein levels, and go on to show that IGFBP-3 antagonizes the growth of 184htert cells in an IGF-dependent manner.

Materials and methods

Cell culture and treatment. 184htert human mammary epithelial cells (obtained from Dr. S. Dunn) were cultured in mammary epithelial cell growth media (MEGM) (BioWhittaker, Walkersville, MD) supplemented with isoproterenol (2.5 µg/ml), transferrin (1 µg/ml), and G418 (400 µg/ml) at 37°C and 5% CO₂.

Celecoxib was obtained from Searle Pharmacia (St. Louis, MO). A stock solution of 50 mM in DMSO was used. Stock solution was added to the test media at dilutions of at least 1:1000 and control samples were treated with vehicle (DMSO). The concentration of DMSO in the test media never exceeded 0.1% (v/v). Human recombinant non-gly-

cosylated IGFBP-3 was obtained from Protigen Incorporation (Mountain View, CA).

Cell proliferation assay. We used a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay to determine cell proliferation (cell viability). Cells (1.5×10^5 /well) were plated in six-well plates in MEGM. For the celecoxib growth experiments, fresh MEGM with celecoxib was added after 24 h. For the IGFBP-3 growth experiments, cells were plated in MEGM for 24 h, washed and incubated in serum-free/phenol red-free (SPF) DMEM/F12 (Invitrogen) or 5%-fetal bovine serum (FBS) containing DMEM-F12 for an additional 24 h, and then treatments were added in fresh media. After the appropriate treatment time (as indicated in figure legends), MTT (Sigma Chemical, St. Louis, MO) was added to a final concentration of 1 mg/ml, the reaction mixture was incubated for 3 h at 37°C. The resulting crystals were dissolved in 0.04% HCl in isopropanol and the absorbance was read at 562 nm.

Microarray analysis of gene expression. Total cellular RNA was isolated from cells by the RNeasy-B method (Tel-Test, Friendswood, TX), according to the manufacturer's protocol. The controls and treatments were performed in triplicate. Gene expression analysis was done using the Affymetrix (Santa Clara, CA) U95 microarray. Biotinylated target cRNA was prepared from total RNA for hybridization to the Probe Arrays. First, double stranded cDNA was synthesized from total RNA using T7-(dT)24 oligo primer. Then an in vitro transcription (IVT) reaction was done to produce the biotin-labeled cRNA from the cDNA. The cRNA was fragmented to 35–200 bp and then added to a hybridization cocktail which contains the probe array controls. It is then hybridized to the oligonucleotide probes on the array for 18 h at 45°C. The probe array is then stained, washed, and scanned.

The data were processed by Affymetrix Microarray Suite 5.0 to derive expression scores. These were truncated at a lower value of 1, then log transformed. The treated and control groups were compared with a *t* test, and the most significant genes ($P < 0.05$) are shown in a scatterplot of fold change. The y-axis is the difference between the average log-transformed values for the treated vs control, which is equivalent to the ratio of the geometric mean expressions for these groups and the x-axis is the average log-transformed expression in controls.

Quantitative real-time PCR (TaqMan). Two micrograms of total RNA was treated with DNase I (Invitrogen) to remove DNA contamination. Then, the RNA was reverse-transcribed using MMLV reverse transcriptase (Invitrogen) in a 50-µl reaction mixture. Taqman primers and probes for quantitative detection of IGFBP-3 and 18S rRNA were designed with Primer Express (ABI-Perkin-Elmer) using the GenBank accession number (X64875 for IGFBP-3 and M10098 for 18S). PCR primer and probe sequences were as follows (all 5' to 3' direction): IGFBP-3: forward CGCCAGCTCCAGGAAATG, reverse GCATGCC CTTTCTTGATGATG, and probe: CAGCAGCACCGGGTGTCT GATC, for an amplicon length of 148 bp; 18S: forward CCGGTAC CACATCCAAGGAA, reverse GCTGGAATTACCGCGGCT, and probe: TGCTGGCACCAGACTTGCCCTC, for an amplicon length of 188 bp. cDNA samples were mixed with 2× Universal TaqMan buffer containing the Taq enzyme, primers at a final concentration of 300 nM for IGFBP-3 and 40 nM for 18S, and probe at a final concentration of 200 nM for IGFBP-3 and 150 nM for 18S in a total volume of 25 µl. The thermal cycler conditions were 50°C 2 min, 95°C 10 min, and 42 cycles of 95°C 15 s and 60°C 1 min. All PCRs and analysis were performed by using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). All samples were run in triplicate.

Western ligand blotting. After 24 h treatment in serum-free/phenol red-free media, conditioned media were collected and concentrated 20-fold using Centricons (Millipore, Bedford, MA). Concentrated conditioned media were subjected to 10% SDS-polyacrylamide gel electrophoresis under non-reducing conditions. Proteins were transferred to nitrocellulose membranes and the membranes were blocked and probed with [¹²⁵I]IGF-I (Perkin-Elmer, Boston, MA) and exposed to X-ray film.

Flow cytometry to assay cell cycle distribution and apoptosis. The treatment conditions for cell cycle analysis were similar to those used

for the MTT assay. After the appropriate treatment period (as listed in the figure legends), adherent cells were collected using trypsin–EDTA and floating cells were collected by centrifugation. The cells were combined and washed twice with ice-cold phosphate-buffered saline (PBS) and fixed in 70% ethanol at -20°C overnight. For cell cycle analysis, the cells were washed twice with ice-cold PBS and resuspended in propidium iodide buffer (PBS, 0.1% Triton X-100, 0.1 mM EDTA, 0.05 mg/ml ribonuclease A, and $50\text{ }\mu\text{M}$ propidium iodide). After 30 min at room temperature, the cell cycle distribution was determined by flow cytometry with a FACSCalibur (Becton–Dickinson, Franklin Lakes, NJ). The proportion of cells in the hypodiploid (sub-G1) area was considered to be apoptotic.

Results and discussion

The 184htert cell line is an immortal breast epithelial cell line that was created by transfecting a retrovirus expressing the human telomerase reverse transcriptase gene into normal breast epithelial cells [11]. In order to confirm that these cells were immortal and not transformed, we grew them in soft agar for over 3 weeks and they did not form colonies, whereas the MCF-7 breast cancer cell line grown in parallel did form colonies (data not shown). This result is consistent with those of Toouli et al. [12] who observed that htert-immortalized breast epithelial cells did not form tumors in nude mice. Therefore, the 184htert cell line is a reasonable system to study the early steps in mammary carcinogenesis. Furthermore, since we detected COX-2 protein expression in these cells (data not shown), 184htert was a suitable cell line to study the chemopreventive effects of the COX-2 inhibitor celecoxib.

To determine if celecoxib could inhibit the growth of these cells, they were treated with either $25\text{ }\mu\text{M}$ or $50\text{ }\mu\text{M}$ celecoxib for up to 48 h. As seen in Fig. 1A, celecoxib inhibited the growth of 184htert cells, with the $50\text{ }\mu\text{M}$ dosage being significantly more potent than the $25\text{ }\mu\text{M}$ dosage ($\sim 30\%$ growth inhibition by $25\text{ }\mu\text{M}$ dose after 48 h, whereas virtually all cells were non-viable after 24 h with the $50\text{ }\mu\text{M}$ dose). The observed difference in the potency between 25 and $50\text{ }\mu\text{M}$ celecoxib was similar to data we had previously obtained with celecoxib treatment of BxPC-3 pancreatic cancer cells [13]. In that system, the difference in potency between the two doses of celecoxib could be attributed to the fact that $50\text{ }\mu\text{M}$ dose induced apoptosis, whereas the $25\text{ }\mu\text{M}$ dose induced G1 arrest. In order to see if this situation was paralleled in 184htert cells, cell cycle analysis was performed on celecoxib-treated cells. After 6 h treatment, $50\text{ }\mu\text{M}$ celecoxib induced apoptosis in 184htert cells ($\sim 15\%$ in sub-G1 population after 6 h treatment, Fig. 1B, panel 3), whereas no apoptosis was observed for cells treated with $25\text{ }\mu\text{M}$ celecoxib (Fig. 1B, panel 2).

Having established that $50\text{ }\mu\text{M}$ celecoxib could inhibit growth and induce apoptosis in 184htert cells, we wished to clarify the mechanism underlying this chemopreventive effect. To do so, differential gene expression was

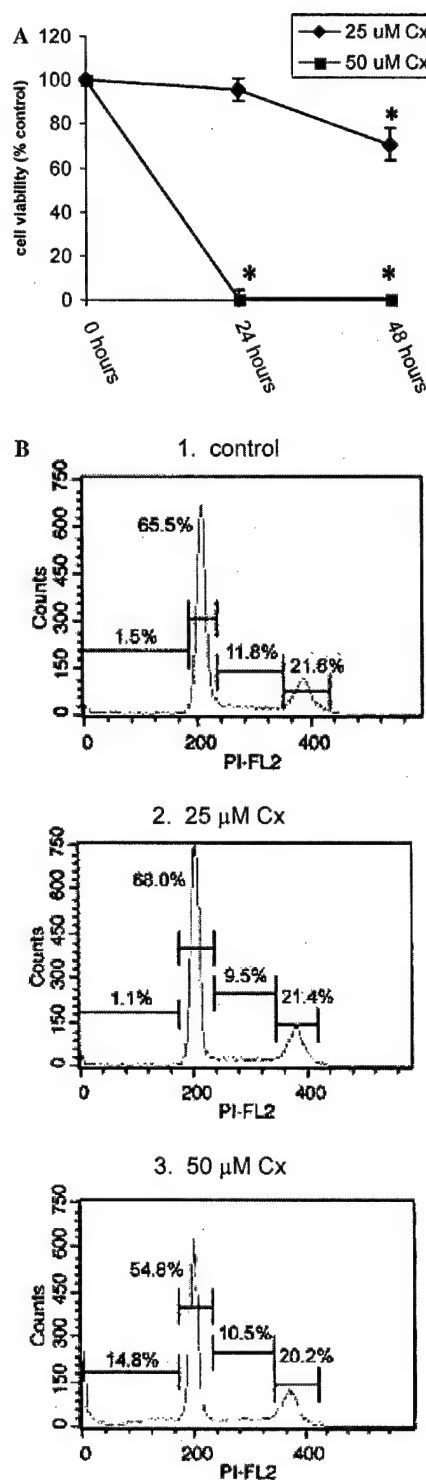


Fig. 1. Celecoxib inhibits growth and induces apoptosis in 184htert cells. (A) Cells were treated for up to 48 h with either $25\text{ }\mu\text{M}$ celecoxib (Cx) or $50\text{ }\mu\text{M}$ Cx in MEGM. Cell viability was determined by the MTT assay and the results are expressed as a percent of the control. Statistically significant changes in cell viability ($P < 0.05$) are marked with *. (B) Cells were treated for 6 h with either 25 or $50\text{ }\mu\text{M}$ Cx in MEGM. Cell cycle analysis was determined using propidium iodide staining and flow cytometry. Percentage of cells in apoptosis (sub-G1), G1, S, and G2/M are indicated.

Table 1
Upregulation of gene expression induced by celecoxib treatment of 184htert cells

GenBank ID	Affymetrix ID	Gene name	Fold change	P value
—	1842_at	Oncogene Tls/Chop, fusion activated	420.5	<0.00001
Z82244	33802_at	Heme oxygenase (decycling) 1	394.9	<0.00001
S62138	39420_at	FUS-CHOP fusion protein [<i>Homo sapiens</i>], mRNA sequence	234.7	<0.00001
AB018287	37483_at	Histone deacetylase 9	228.2	<0.00001
M36820	37187_at	Chemokine (C-X-C motif) ligand 2	59.7	<0.00001
M28130	1369_s_at	Interleukin 8	53.0	<0.00001
N35832	34964_at	H3 histone family, member B	43.0	<0.00001
X04430	38299_at	Interleukin 6 (interferon, β 2)	39.0	0.00024
AL038340	32243_g_at	Crystallin, α B	32.9	0.00013
M36821	34022_at	Chemokine (C-X-C motif) ligand 3	32.5	0.00002
U04636	1069_at	Cyclooxygenase-2	31.0	<0.00001
M17017	35372_r_at	Interleukin 8	28.9	0.00014
X51757	35965_at	Heat shock 70 kDa protein 6 (HSP70B')	22.0	0.00002
M35878	37319_at	Insulin-like growth factor binding protein 3	20.7	<0.00001
U78722	33572_at	Zinc finger protein 165	20.6	<0.00001
U83981	37028_at	Protein phosphatase 1, regulatory (inhibitor) subunit 15A	20.5	<0.00001
U10550	37279_at	GTP binding protein overexpressed in skeletal muscle	19.1	<0.00001
AL038340	32242_at	Crystallin, α B	18.8	0.00006
M31166	1491_at	Pentaxin-related gene, rapidly induced by IL-1 β	18.6	0.00048
M35296	1084_at	V-abl Abelson murine leukemia viral oncogene homolog 2 (arg, Abelson-related gene)	17.9	<0.00001

Due to space restrictions, only the top 20 changes detected are listed. Complete list of detected upregulations ($P \leq 0.001$) are listed in supplementary data.

Table 2
Downregulation of gene expression induced by celecoxib treatment of 184htert cells

GenBank ID	Affymetrix ID	Gene name	Fold change	P value
U33761	1941_at	S-phase kinase-associated protein 2 (p45)	0.06	0.00004
D16532	36873_at	Very low density lipoprotein receptor	0.06	0.00011
AF029893	33140_at	UDP-GlcNAc: β Gal β -1,3- <i>N</i> -acetylglucosaminyltransferase 6	0.07	0.00015
X05610	36659_at	Collagen, type IV, α 2	0.09	0.00002
X76388	39355_at	ATP-binding cassette, sub-family E (OABP), member 1	0.1	<0.00001
X68836	32571_at	Methionine adenosyltransferase II, α	0.11	0.00004
AL040137	41807_at	ESTs	0.12	0.00031
M60047	38489_at	Heparin-binding growth factor binding protein	0.12	<0.00001
AA868382	32773_at	Major histocompatibility complex, class II, DQ α 1	0.12	<0.00001
U44105	161_at	RAB9, member RAS oncogene family, pseudogene 1	0.12	<0.00001
AA426361	39538_at	Sortilin 1	0.15	0.00018
J03589	923_at	Ubiquitin-like 4	0.15	0.00002
L34041	33902_at	Glycerol-3-phosphate dehydrogenase 1 (soluble)	0.16	<0.00001
M37712	33299_at	P58/GTA protein kinase [<i>Homo sapiens</i>], mRNA sequence	0.16	0.00044
U07225	36292_at	Purinergic receptor P2Y, G-protein coupled, 2	0.16	0.00073
M69040	36099_at	Splicing factor, arginine/serine-rich 1 (splicing factor 2, alternate splicing factor)	0.16	0.00024
M81933	1738_at	Cell division cycle 25A	0.17	0.00057
U54617	36739_at	Pyruvate dehydrogenase kinase, isoenzyme 4	0.17	0.00032
AL031588	41660_at	Cadherin, EGF LAG seven-pass G-type receptor 1 (flamingo homolog, <i>Drosophila</i>)	0.18	0.00027
AJ004832	34874_at	Neuropathy target esterase	0.18	0.00015

Due to space restrictions, only the top 20 changes detected are listed. Complete list of detected downregulations ($P \leq 0.001$) are listed in supplementary data.

evaluated by comparing 184htert cells treated with or without 50 μ M celecoxib for 6 h using the Affymetrix U95 cDNA microarray. Results from the microarray analysis of celecoxib-induced changes in gene expression are listed in Tables 1–3 and supplementary data (available online). Evidence for the validity of the gene expression data includes the strong upregulation of

COX-2 expression by celecoxib treatment (\sim 30-fold induction, Table 1), an observation that we confirmed at the protein level (data not shown) and that has been reported in other systems using various NSAIDs [14,15].

Our laboratory is particularly interested in the association between IGF physiology and breast cancer as IGF-I is known to be a potent mitogen for breast

Table 3

Expression changes induced by celecoxib treatment of 184htert cells for genes involved in the induction of apoptosis.

GenBank ID	Affymetrix ID	Gene name	Fold change	P value
AB011421	37524_at	Serine/threonine kinase 17b (apoptosis-inducing)	7.9	0.00003
AJ006288	38871_at	B-cell CLL/lymphoma 10	3.0	0.00008
U03106	2031_s_at	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	2.9	0.00003
AF005775	1868_g_at	CASP8 and FADD-like apoptosis regulator	2.7	0.00079
AF032886	34740_at	Forkhead box O3A	2.3	0.00002
M77142	33852_at	TIA1 cytotoxic granule-associated RNA binding protein	2.0	0.00011

Genes listed displayed statistically significant expression changes ($P \leq 0.001$) after celecoxib treatment and their Gene Ontology attributes include induction of apoptosis.

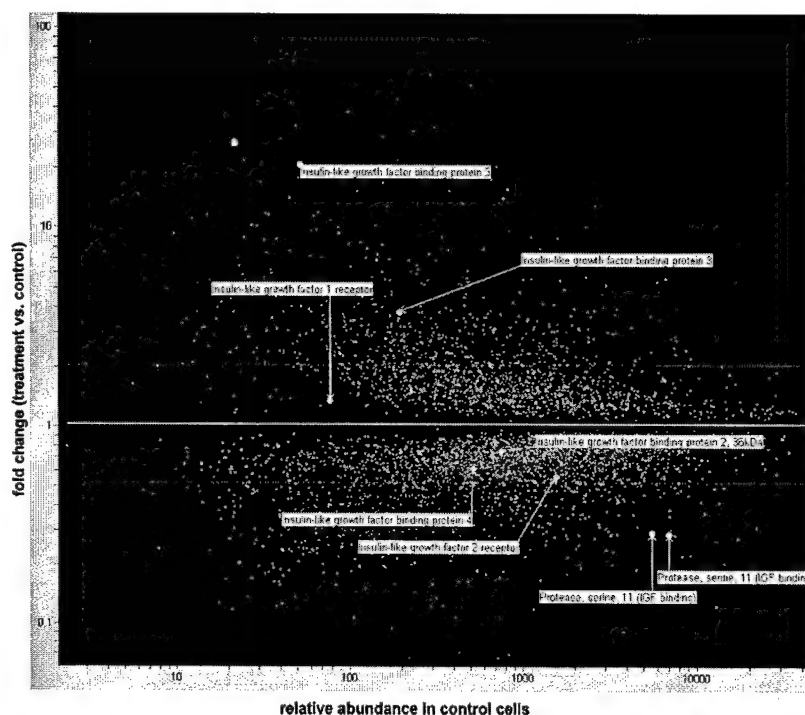


Fig. 2. Celecoxib-induced changes in the expression of genes associated with IGF physiology. 184htert cells were treated with DMSO control or 50 μ M celecoxib for 6 h in MEGM, and then total RNA was collected and sent for microarray analysis as described in Materials and methods. Probes which detected a statistically significant change in gene expression induced by celecoxib treatment ($P < 0.05$) are displayed as dots in the scatterplot, and probes that correspond to genes which are known to be involved in IGF physiology are labeled.

epithelial cells [16], and serum IGF-I levels have been positively correlated with pre-menopausal breast cancer risk [17]. Fig. 2 is a scatterplot of the microarray data where each dot represents a probe that detected a statistically significant change in gene expression induced by celecoxib treatment ($P < 0.05$). Probes that correspond to genes whose products are involved in IGF physiology are labeled. A treatment/control signal ratio (fold change) of ≥ 2 or ≤ 0.5 was considered as a significant induction or repression, consistent with previous reports of microarray gene expression analysis [18,19]. Our array data show that celecoxib did not significantly alter ($0.5 > \text{fold change} < 2.0$) the expression of the IGF-I receptor, IGF-II receptor, nor IGFBPs-2 and 4 (Fig. 2). Furthermore, celecoxib-induced

changes in the expression of the ligands IGF-I and IGF-II, and IGFBPs-1,5,6,7 were not statistically significant ($P < 0.05$, data not shown). However, as seen in Fig. 2, the expression of IGFBP-3 was significantly upregulated by celecoxib treatment (~ 20 -fold induction by hybridization to the 37319_at probe and ~ 4 -fold induction by hybridization to the 1586_at probe). IGFBP-3 is the major circulating IGFBP and it has been shown to inhibit IGF-induced growth and also to have IGF-independent growth inhibitory effects [9,10]. Therefore, we hypothesized that IGFBP-3 may be an important modulator of celecoxib's chemopreventive effects. Consistent with this hypothesis, two different probes detected a ~ 0.3 -fold decrease in the expression of protease, serine, 11 (IGF binding) (Fig. 2). This protein, which is

also known as L56 and HtrA1, has been suggested to be a protease which cleaves and inactivates IGFBPs [20]. Thus, celecoxib appears to not only upregulate IGFBP-3 mRNA abundance, but it also decreases the expression of an IGFBP protease.

In order to confirm the induction of IGFBP-3 mRNA by celecoxib, quantitative real-time RT PCR was performed on untreated and celecoxib-treated cells. As seen in Fig. 3A, a ~3-fold induction in IGFBP-3 mRNA was detected after 6 h treatment with 50 μ M celecoxib. This experiment was performed in serum-free, phenol-red free (SPF) media in order to prevent other factors (estrogen, for example) from interfering with IGFBP-3 promoter activity. To determine if the celecoxib-induced increase in IGFBP-3 mRNA correlated to an increase in IGFBP-3 protein, conditioned media from 184htert cells treated or untreated with celecoxib were assayed by [125 I]IGF-I Western ligand blotting. As seen in Fig. 3B, we detected a weak doublet band at ~40–45 kDa in SPF

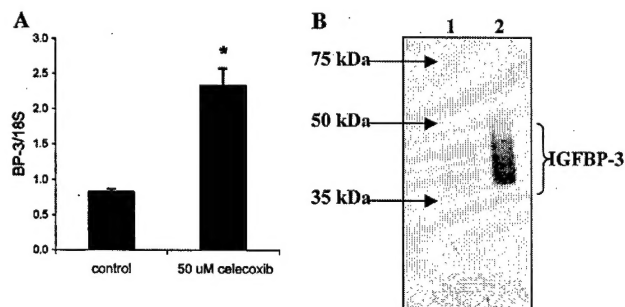


Fig. 3. Celecoxib induces IGFBP-3 mRNA and protein expression in 184htert cells. (A) Total RNA collected from 184htert cells treated with DMSO control or 50 μ M celecoxib for 6 h in SPF media was analyzed by TaqMan as described in Materials and methods (*statistically significant change compared to control, $P = 0.01$). (B) Concentrated conditioned media from 184htert cells treated with DMSO control (lane 1) or 50 μ M celecoxib (lane 2) for 24 h in SPF media were separated by 10% SDS-polyacrylamide gel electrophoresis and probed by [125 I]IGF-I ligand blotting as described in Materials and methods.

conditioned media under control conditions for 24 h, and treatment with 50 μ M celecoxib resulted in a significant increase in the intensity of this doublet. Therefore, celecoxib treatment of 184htert cells is associated with upregulation of both IGFBP-3 mRNA and protein. The induction of IGFBP-3 by celecoxib is consistent with the results of Pold et al. [21], who have shown that IGFBP-3 mRNA and protein levels were significantly decreased in A549 human lung cancer cells transduced with COX-2 when compared to the wild-type cell line. Taken together, these data lead to the working hypothesis that IGFBP-3 expression is inversely correlated with COX-2 activity.

Having established that celecoxib upregulates IGFBP-3 expression, we wished to determine if IGFBP-3 could inhibit the growth of 184htert cells. Published results on the effect of IGFBP-3 on non-malignant breast epithelial cell growth have been inconsistent. Martin and Baxter [22] have shown that doses ranging from 10 to 100 ng/ml of IGFBP-3 can inhibit the growth of the immortal breast epithelial cell line MCF-10a in an IGF-independent fashion, however, McCaig et al. [23] have demonstrated a biphasic response of MCF-10a cells to IGFBP-3 under serum-free conditions, with low doses (~20 ng/ml) acting as growth inhibitors and high doses (100–200 ng/ml) acting as growth promoters. Furthermore, Strange et al. [16] have shown that 50–500 ng/ml IGFBP-3 can inhibit both IGF-dependent and -independent growth of normal human mammary epithelial cells in a dose-dependent manner.

When we treated 184htert cells with 500 ng/ml IGFBP-3 for 72 h in SPF media, no significant effect on cell growth was observed (Fig. 4A). Treatment with lower doses of IGFBP-3 (25–100 ng/ml) for up to 72 h yielded similar results (data not shown), suggesting that 184htert cells do not display any IGF-independent growth inhibition by IGFBP-3. The fact that we did not observe the IGF-independent effects of IGFBP-3 that

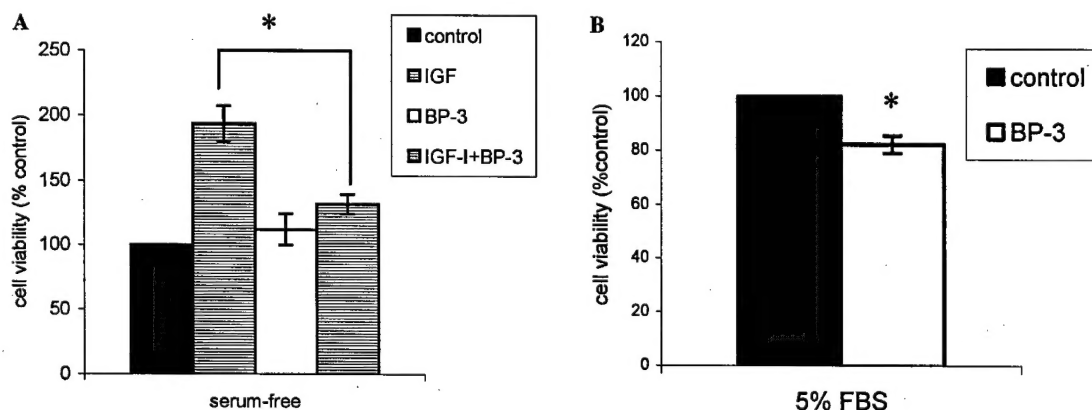


Fig. 4. IGFBP-3 antagonizes IGF-I-induced growth. MTT assay of 184htert cells treated for 72 h in SPF (A) or 5% FBS-containing media (B) with 50 ng/ml IGF-I alone, 500 ng/ml IGFBP-3 alone, or both in combination (as indicated in the figure legend). Statistically significant changes in cell viability ($P < 0.05$) are marked with *.

were reported in other breast epithelial cell lines might be due to the transfected telomerase in 184hTert cells, as telomerase activity in breast epithelial cells has been associated with resistance to at least two other growth inhibitors, namely topoisomerase inhibitors [24] and transforming growth factor β [25]. It should be noted that our array data detected a celecoxib-induced 0.3-fold decrease in the expression of telomerase reverse transcriptase ($P = 0.0003$, supplementary data), potentially antagonizing its protective effect against growth inhibition by celecoxib. This may explain why celecoxib is such a potent growth inhibitor in this system.

Since IGFBP-3 did not affect the growth of 184hTert cells under serum-free conditions, its upregulation is most likely not directly contributing to the celecoxib-induced growth inhibition observed in Fig. 1A. However, upregulation of IGFBP-3 by celecoxib may contribute to its chemopreventive action by suppressing mitogenic and anti-apoptotic signals induced by IGF-I. To test this hypothesis, 500 ng/ml IGFBP-3 was added to 184hTert cells in the presence of 50 ng/ml IGF-I. As seen in Fig. 4A, 500 ng/ml IGFBP-3 was capable of significantly attenuating an IGF-I-induced increase in cell growth ($\sim 70\%$ decrease, IGFBP-3 and IGF-I vs IGF-I alone). Furthermore, when 500 ng/ml IGFBP-3 was added to cells growing in 5% fetal bovine serum (FBS), $\sim 20\%$ growth inhibition was observed (Fig. 4B), potentially due to IGFBP-3 blocking the actions of the IGF-I present in FBS. These data indicate that IGFBP-3 can significantly attenuate IGF-induced growth in 184hTert breast epithelial cells, which is consistent with published results using primary breast epithelial cells [16].

Studies are ongoing to evaluate the mechanism by which celecoxib induces IGFBP-3 expression. A closer look at the IGFBP-3 promoter reveals a cluster of 11 p53 consensus binding sites, five consensus Yin and Yang-1 sites, a cluster composed of an Sp1/GC-rich site, two consensus independent AP-2 sites overlapping a GA-box, and a putative p300 DNA-binding site, all 5' to a TATA box [26]. Examining the transcriptional activators modified by celecoxib treatment in our microarray experiment (supplementary data) revealed a 3.1-fold increase ($P = 0.00003$) in the expression of a protein called CBP/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2 (CITED2). It has been recently suggested that interactions among AP-2, CITED2, and p300/CBP are necessary for AP-2 mediated transcriptional activation [27], and since both AP-2 and p300 binding sites are located within the IGFBP-3 promoter, celecoxib-induced IGFBP-3 expression may be at least partially due to an upregulation of CITED2.

In this report, we show that the COX-2 inhibitor celecoxib can inhibit growth and induce apoptosis in 184hTert breast epithelial cells. Gene expression analysis using cDNA microarrays revealed that the effects of

celecoxib are associated with expression changes in many genes of interest, consistent with the view that COX-2 inhibitors have multiple mechanisms of action as cancer chemopreventive agents. Our array data demonstrated that celecoxib induced the expression of IGFBP-3, an observation we confirmed at the mRNA and protein levels. The fact that IGFBP-3 can attenuate the growth promoting effects of IGF-I in this experimental system raises the possibilities that: (1) celecoxib may decrease breast cancer risk by a mechanism involving IGFBP-3 induction, acting to slow proliferation of at-risk breast epithelial cells, and (2) the risk reduction associated with COX-2 inhibitors may not be homogeneous in populations, but rather be particularly useful in individuals with higher serum IGF-I levels.

Acknowledgments

We thank Dr. Sandra Dunn (University of British Columbia) for the 184hTert cell line, Laurence Lejeune and Dr. Anne Camirand for their technical assistance with the flow cytometer, and Uzma Majeed for manuscript preparation.

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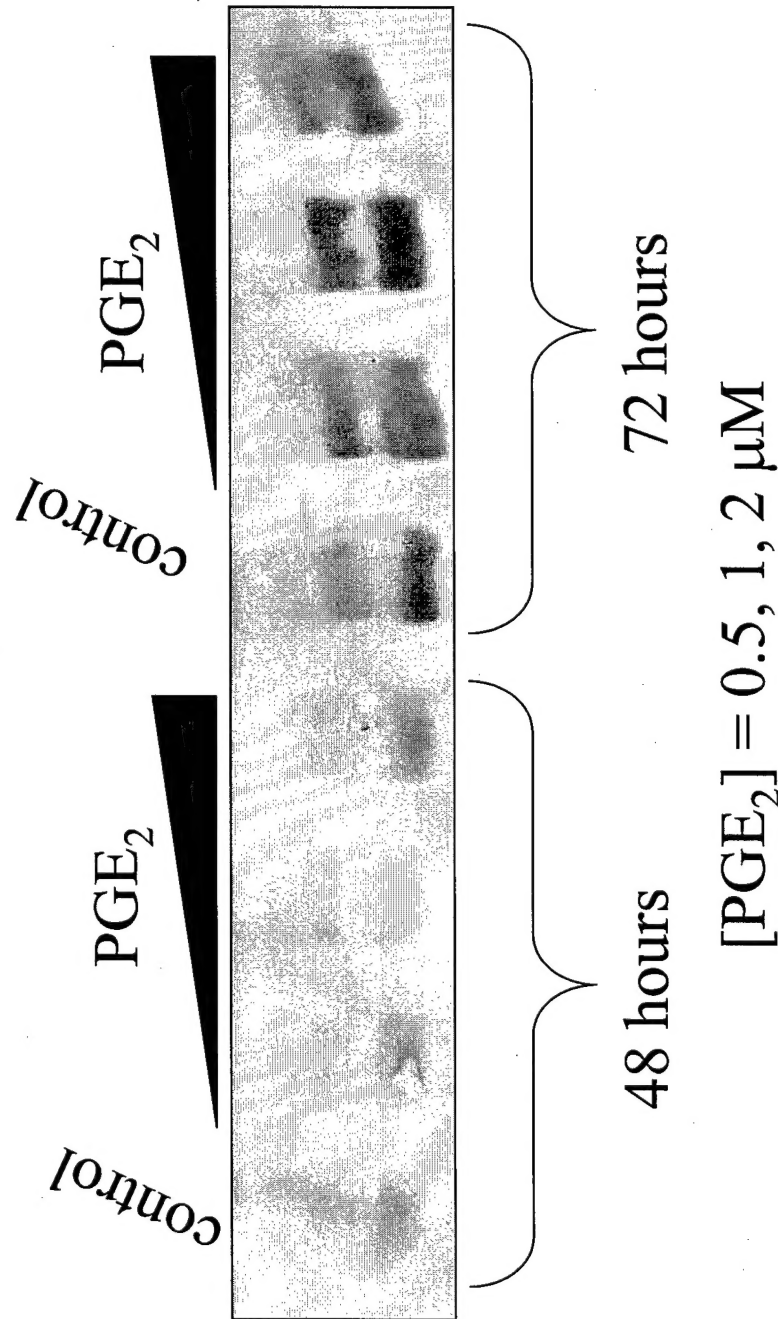


Figure 5: PGE₂ has no effect on IGFBP-3 expression in 184htert cells. The 184htert breast epithelial cell line was treated with various concentrations of PGE₂ (as indicated) and conditioned media was collected at 48 and 72 hours. The conditioned media was concentrated 20-fold, separated on a 10% SDS-PAGE, and then IGFBP-3 expression was analyzed by western blotting.